

NEW BODIPY-CHOLESTERYL OLEATE ANALOG IN HIGH-DENSITY LIPOPROTEIN ENVIRONMENT

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Kolesterolin aineenvaihdunnan ja toiminnan tutkiminen on olennaista eri tautiprosessien ymmärtämiseksi ja uudenlaisten hoitomenetelmien kehittämiseksi. Kaikki lipidipohjaiset markkerimolekyylit häiritsevät ympäristönsä normaalia toimintaa. Tavoitteena on löytää markkerimolekyylejä, jotka aiheuttavat mahdollisimman vähän häiriöitä ja toisaalta ovat riittävän herkkiä seurattaviksi kokeellisilla menetelmillä.

Tässä työssä tutkitaan ominaisuuksiltaan lupaavaa fluoresoivaa BODIPY-markkeria kytkettynä kolesteroliolaattiin (BODIPY-CE). Aiemmissa tutkimuksissa BODIPY-kolesterolin on havaittu käyttäytyvän hyvin kolesterolin kaltaisesti. Tiedossa ei ole aikaisempia tutkimuksia BODIPY-CE:n ominaisuuksista ja käyttäytymisestä tai soveltuvuudesta markkerimolekyyliksi lipoproteiini ympäristössä.

BODIPY-CE:n ominaisuuksia ja käyttäytymistä tutkitaan tässä työssä molekyyliidynamiikkaan perustuvilla tietokonesimulaatioilla. Simulaatiot mahdollistavat lyhyillä aikaskaaloilla ja nanometrin mittaluokassa tapahtuvien ilmiöiden tutkimisen. BODIPY-CE:n käyttäytymistä tutkitaan yksinkertaistetussa HDL-partikkelissa, joka koostuu ApoA-I proteiineista ja kahden eri lipidin muodostamasta lipidiosasta.

Tutkimustulosten perusteella BODIPY-CE:n käyttäytyminen muistuttaa normaalia kolesteroliolaattia. Pieniä eroja havaitaan intramolekulaarisissa rakenteissa ja siinä, että BODIPY-CE häiritsee hieman ympäröivien kolesteroliolaattimolekyylien järjestäytymistä. BODIPY-CE:n diffuusio on normaalia kolesteroliolaattia hitaampaa, ero on sitä suurempi, mitä kauempana ytimestä molekyylit sijaitsevat.

Tulosten perusteella BODIPY-CE vaikuttaa lupaavalta markkerimolekyyliltä kolesteroliaineenvaihdunnan tutkimiseen. Lisätutkimuksia tarvitaan BODIPY-CE:n ominaisuuksien tutkimiseksi pidemmällä aika- ja pituusskaaloilla.

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1 Introduction

Cholesterol is an essential structural component of eukaryotic cell membranes. It affects the properties of cell membranes by affecting the organization of the other lipids in the membrane. Membrane permeability and stiffness are some of the properties affected by cholesterol concentration (1, 2). Cholesterol is also an important component of bile acids, steroid hormones and fat-soluble vitamins. In addition, cellular sterol distribution and trafficking are key players in the development of many diseases (3). Cholesterol is an important player in the development of atherosclerosis, and elevated plasma cholesterol levels are sufficient alone to drive the development of atherosclerosis (4, 5, 6).

Cholesterol can be obtained from two sources: diet or de novo biosynthesis. Most of the cholesterol utilized by the body is endogenously synthesized. The body has enough synthesizing capacity even if there is no cholesterol from diet. A major site for biosynthesis of cholesterol is the liver, but significant amounts of cholesterol is also synthesized in intestine, adrenal glands and reproductive organs (7). Tracking cholesterol is important, and development of new tools and methods for this purpose is crucial to progress in this area. The problem with all lipid based probes that are different from endogenous lipids is that they disturb the system to some extent. Therefore, the aim is to find probes that are less harmful and at the same time have sufficient sensitivity for tracking. For studying cholesterol and its derivatives, an optimal probe would have as cholesterol-like properties as possible.

Many different compounds mimicking cholesterol have been constructed, but they have limitations. Using nitrobenzoxadiazole (NBD) and dipyrromethene difluoride (BODIPY) fluorophores some of the properties of cholesterol are successfully matched, but the membrane partitioning was found to be opposite to cholesterol (8). Dehydroergosterol (DHE) has been a popular fluorophore since its behavior is very similar to normal cholesterol, but its fluorescence properties are not optimal: too rapid photobleaching, and emission and excitation wavelengths in the ultraviolet region (8). NBD and BODIPY fluorophores and DHE cholesterol have been used in fluorescent cholesterol ester (CE) analogs in various studies, but the impact of fluorophores has not been previously characterized (8).

Recently a compound with a BODIPY moiety linked to cholesterol's carbon-24 was found to have promising features, and in other studies it has been shown to closely mimic membrane partitioning and trafficking of cholesterol (8, 9, 10, 11, 12). To our knowledge, the behavior of its derivatives (like cholesteryl oleate) and its properties in lipoprotein-like environments have not been characterized before. In this study, through atomic scale simulations we analyze the properties and the behavior of BODIPY-cholesteryl oleate (BODIPY-CE, Figure 1), based on the recent cholesterol analog, in an HDL-like environment, and characterize its suitability for cholesterol tracking in lipoprotein-like

environments.

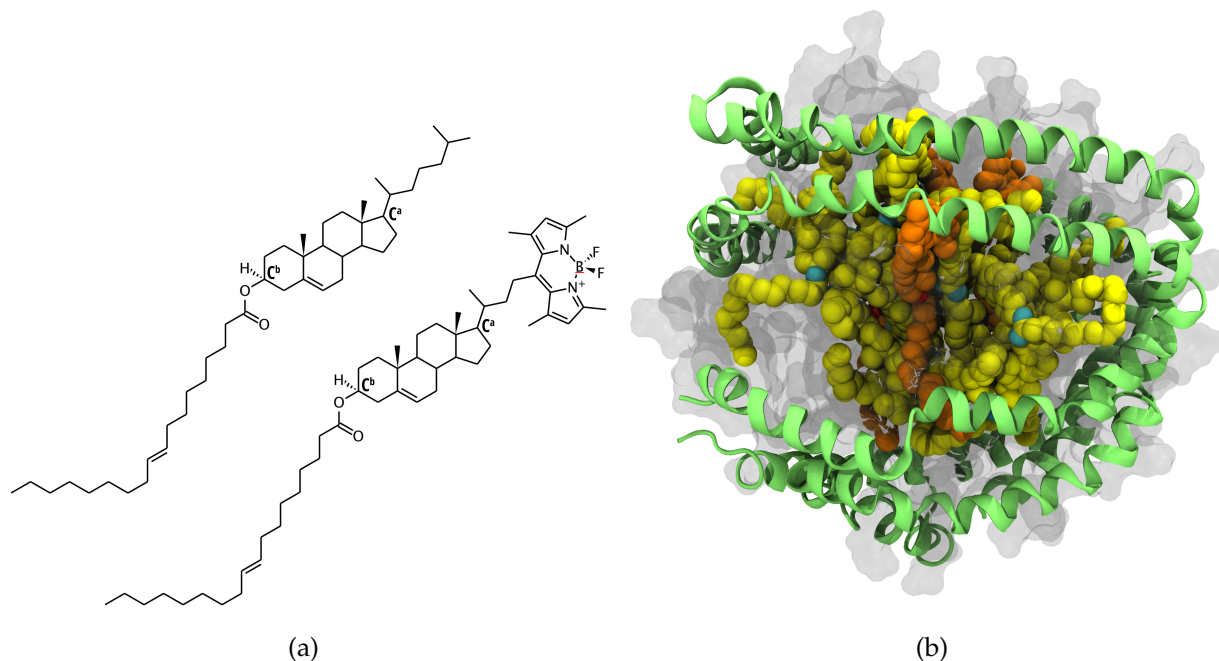


Figure 1: (a) Chemical structures of BODIPY-cholesteryl oleate (BODIPY-CE, lower panel) and normal cholesteryl oleate (CE, upper panel). C^a and C^b are atoms of the ring structure that define the director of the ring structure, see 2.2 for details. (b) Snapshot of the system 01 after simulating for 100 ns. CE is yellow with cyan oxygen atoms. BODIPY-CE is orange with red oxygen. Transparent grey is for the phospholipids and green for the ApoA-I.

2 Methods

2.1 Simulation Methods

This study consists of four different systems for the high density lipoprotein (HDL) particle: a reference system without BODIPY-probes, and three systems in which BODIPY probes were present. Below we describe the main stages of the system setup.

The initial structure for the HDL particle was obtained from an earlier study (13). The HDL particle consists of two ApoA-I, 56 POPC and 16 cholesteryl oleate (CE) molecules solvated with water molecules. This structure was then simulated for a period of 100 ns and used as a reference system.

Using the initial structure, three systems (01, 02, 03) containing BODIPY probes (BODIPY-CE) were constructed. In each system three cholesteryl oleate molecules were chosen randomly and their short acyl chains were manually replaced by a BODIPY probe. After that each BODIPY system was energy minimized and simulated for 100 ns. Three different systems were used to obtain better statistics for BODIPY probes.

Lipid molecules were described using the standard united atom force field parameters (Berger lipids) (14). The cholesteryl oleate force field was obtained from Artturi Koivuniemi (15). The description for the BODIPY probe was adapted from an earlier study and used with Berger lipids (10). For ApoA-I protein molecules we used the all-atom OPLS protein force field (16), and for water we employed the simple point charge (SPC) model (17). Tieleman et al. have reparameterized the dihedral potentials to combine the united atom force field with an all-atom force field (18). In this study a simpler and more inclusive alternative method was used for combining force fields (19).

The molecular dynamics simulations were performed with the GROMACS 4.0 package (20). A time step of 2.0 fs was used for integrating the equations of motion. The van der Waals interactions were calculated up to a cut-off radius of 1.4 nm and the Particle-Mesh-Ewald (PME) technique (21, 22) was utilized for the long-range Coulombic forces. The Nose-Hoover thermostat (23, 24) and the Parrinello-Rahman barostat (25) were used to ensure proper NpT conditions ($T = 310$ K, $p = 1$ atm). Protein and non-protein molecules were coupled to separate thermostats, and the whole system was coupled to the barostat isotropically. A time constant of $\tau = 0.5$ ps was used for the thermostat and time constant of $\tau = 5.0$ ps for the barostat.

2.2 Analysis Methods

The orientational ordering of CE and BODIPY-CE ring structures is studied using an order parameter that measures how well vectors, defined from a molecule, are aligned as a function of distance. This is defined as the average of the second-order Legendre polynomial:

$$S_{RR} = \frac{1}{2} \langle 3 \cos^2 \theta - 1 \rangle, \quad (1)$$

where θ is the angle between two vectors (see below for details). The order parameter S_{RR} gains the value 1 if every vector is aligned in the same direction and 0 if the alignment is random. A vector, called the director, describes the orientation of the ring structure of CE and BODIPY-CE is defined as a vector pointing from the C^a atom to the C^b atom (see Figure 1).

The dynamics of the core lipids is considered in terms of diffusion. Usually diffusion of molecules is studied using mean-square displacements in the long-time limit (31). Since diffusion in this work takes place in a confined environment, it is preferable to use a different method.

In this work diffusion is characterized by considering the lipid center-of-mass position displacement distribution functions over a fixed time t , i.e., jump length distributions. It is

assumed that the centers-of-mass of molecules follow a random walk. The two- and three-dimensional diffusion models can be fitted to these distributions, yielding diffusion coefficient for the given time interval. It is expected that the diffusion coefficient levels off at long times, indicating diffusive behavior to approach the hydrodynamic long-time limit. Two- or three-dimensional diffusion model is used based on which fits better to the displacement distribution. Inside the droplet the space available for diffusion is confined. To avoid artifacts due to this restriction, it is possible to get an estimate for time interval that can safely be used. In an earlier study it was estimated to be about 75 ns (32). In this work a smaller period of 10 ns was used. The method is explained in more detail as part of an earlier study (32).

3 Results

3.1 Equilibration

To verify equilibration of the simulation systems we computed the root mean square displacement (RMSD) as a function of time for protein alpha carbons, shown in Figure 2. RMSDs for the reference system and systems 01 and 03 show a rapid increase until 20 ns, after which a plateau forms at 30 ns. System 02 shows a constant increase, not showing a plateau during the simulation.

Based on the findings above, the following analysis and discussion will focus on the time interval 30 - 100 ns for each system if not mentioned otherwise.

3.2 Overall Structure of the Particles

Radius of gyration for the reference system is 2.77 ± 0.02 nm. Values of 2.76 ± 0.01 nm, 2.73 ± 0.01 nm, and 2.75 ± 0.02 nm were measured for the radius of gyration for the

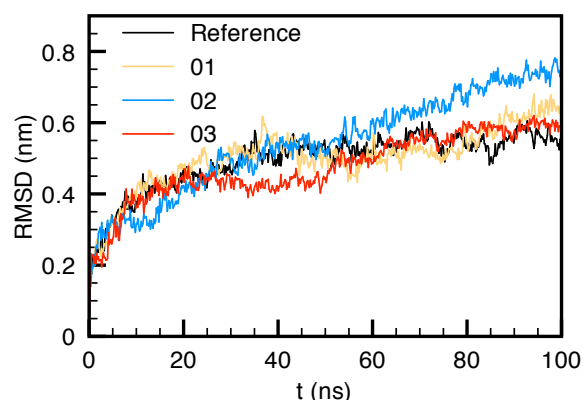


Figure 2: Root mean square deviation of the ApoA-I alpha carbons for each system.

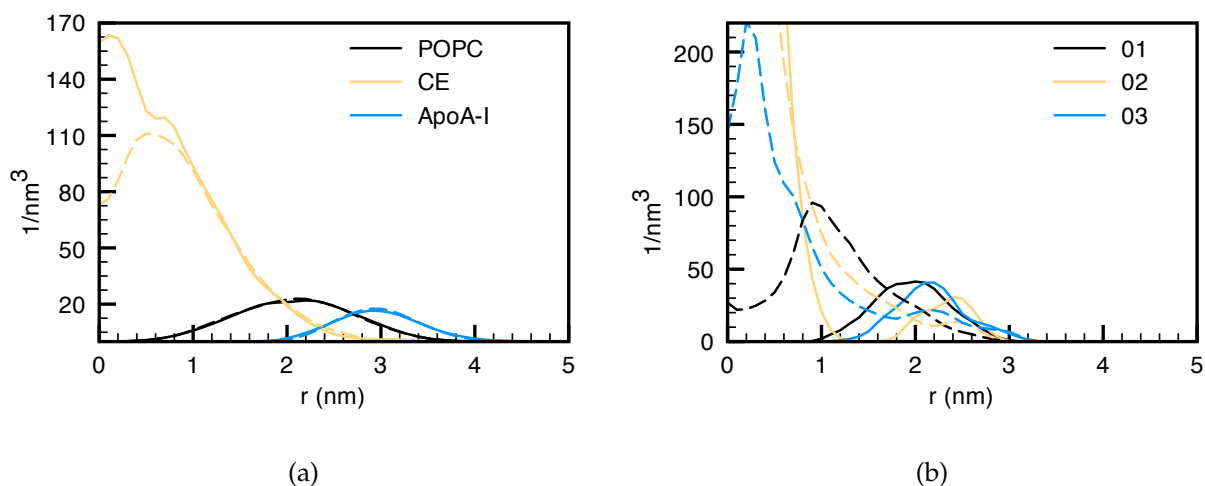


Figure 3: Radial density distributions. (a) Radial density distributions for POPC, CE and the protein in the reference system (solid line), and the average of the three BODIPY-systems (dashed line). (b) Radial density distributions of the BODIPY-probes (solid line) and BODIPY-CE molecules (dashed line) shown for each system separately. In Panel (b) the yellow curve goes up to 850.

BODIPY-systems 01, 02 and 03, respectively. These results and also the average size of the droplet is in good agreement with previous results from both experimental and simulation studies (13, 26). The shape of the particles can be characterized as a prolate spheroid (data not shown) as seen in the previous study (13), but the BODIPY-containing particles are slightly less prolate and resemble more a sphere.

Figure 3 shows the radial density distributions functions (RDFs) for all systems. The molecules in the reference system are organized as expected: Apo-AI on the surface with the polar lipids, and the hydrophobic CE in the core, the latter perturbing slightly the acyl chain region of the lipids. This compares reasonably well with the results from an earlier study regardless that the protein part was not included (27). Distribution of the molecules in the BODIPY-containing systems follows the distribution in the reference system. BODIPY-CE organizes similarly to the normal CE, slightly favoring the central region of the droplet. In BODIPY-systems 01 and 03 the probes are on average located closer to the surface whereas in the system 02 the probes are located deeper in the core of the particle. RDFs for CE in BODIPY-systems are shown in Figure 4 and it is evident that the BODIPY-probes do affect the packing of CEs only minimally on a larger scale. In the next section we will discuss the effects on intramolecular level and later also on an intermolecular level.

Due to the fact that the timescales required for BODIPY-CEs and normal CEs to diffuse the length of the particle diameter are out of reach of these simulations, we will use the systems 01 and 03 to characterize the behavior and effects of BODIPY-CE closer to the surface and the system 02 to consider effects deeper in the core. In practice we will compute averages over the systems 01 and 03 and compare those results with the reference system and the system 02.

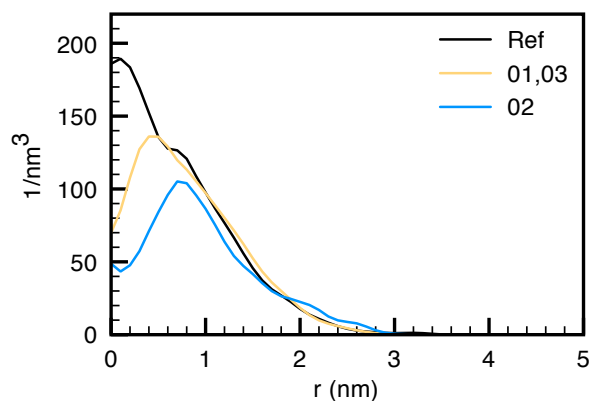


Figure 4: CE radial density distribution function. RDF for CE is shown separately for the reference and the 02 systems. For systems 01 and 03, a common average is shown. Blue curve describes the distribution when probes are located more in the core, and yellow the situation when probes are closer to the droplet surface.

3.3 Intramolecular properties

Figure 5 shows the angle distributions for the oleate chain of both CE and BODIPY-CE. The reference system and also the average over the systems 01 and 03 show similar behavior as observed in earlier studies for an isotropic system and a mixture of POPC and cholesteryl oleate (27, 28). The conformations of CE molecules in BODIPY-systems closely follow the conformations in the reference system, although for an unknown reason in the system 02 there is a peak at 110 degrees that is absent in other systems. BODIPY-CE in the systems 01 and 03 acts similarly to CE with a small difference. In the system 02, BODIPY-CE conformations seem to favor more extended conformations. In an experimental ^{13}C NMR study of unsaturated CEs (29) and in scattering experiments of saturated CEs in isotropic fluid phase (30), it was seen that the oleate tail of CE prefer extended conformations.

The short acyl chain of CE in the reference system and in BODIPY-systems behaves

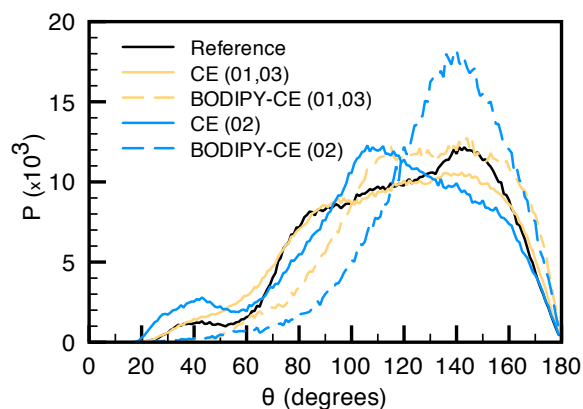


Figure 5: Oleate tail orientation. Angle θ is the one between the ring structure director and the oleate chains start and end points. Data is shown separately for the reference system, average from the systems 01 and 03, and for the system 02.

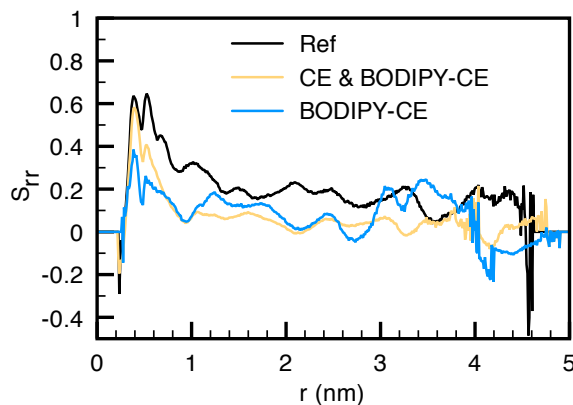


Figure 6: S_{rr} averaged over all the BODIPY-CEs and over all the systems. Black curve shows the results in the reference systems, yellow curve the BODIPY-systems when all BODIPY- and normal CEs are treated the same. Blue curve describes the packing around the BODIPY-CEs. r is the distance between the centers of the directors.

similarly, and prefers almost fully extended conformations (data not shown). Also the BODIPY-probe seems to adopt conformations that mimic the short acyl chain behavior. These results are in good agreement with the earlier bulk-CE simulation (28).

3.4 Intermolecular ordering of CE and BODIPY-CE

The intermolecular orientational order parameter S_{RR} between the directors of CEs is determined in a manner similar to that previously described for an isotropic CE system (28). The S_{RR} describes how the orientation of the ring structures is correlated as a function of distance, i.e., how they are packed.

Figure 6 shows the S_{RR} profiles that are averaged over the systems and Figure 7 shows data separately for each system. The results indicate that at short intermolecular distances, the ring structures (both CE and BODIPY-CE) prefer to stack. However, the correlation disappears at longer distances which indicates a liquid phase. The same kind of behavior is seen in the earlier simulation studies (27, 28).

Based on the results it is evident that BODIPY-CE has a lower tendency to stack and induce stacking around it when compared to CE. The first peak in the S_{RR} profile is almost conserved but the second peak is more suppressed. The larger size of the BODIPY-probe compared to the normal short acyl chain might inhibit the stacking around BODIPY-CE molecules and thus explain the differences seen in the S_{RR} profiles.

3.5 Dynamics of Core Lipids Characterized by Diffusion

In an earlier study it was observed that the rate of diffusion varies significantly inside a lipid droplet as a function of distance from the droplet center-of-mass (32). Because of that,

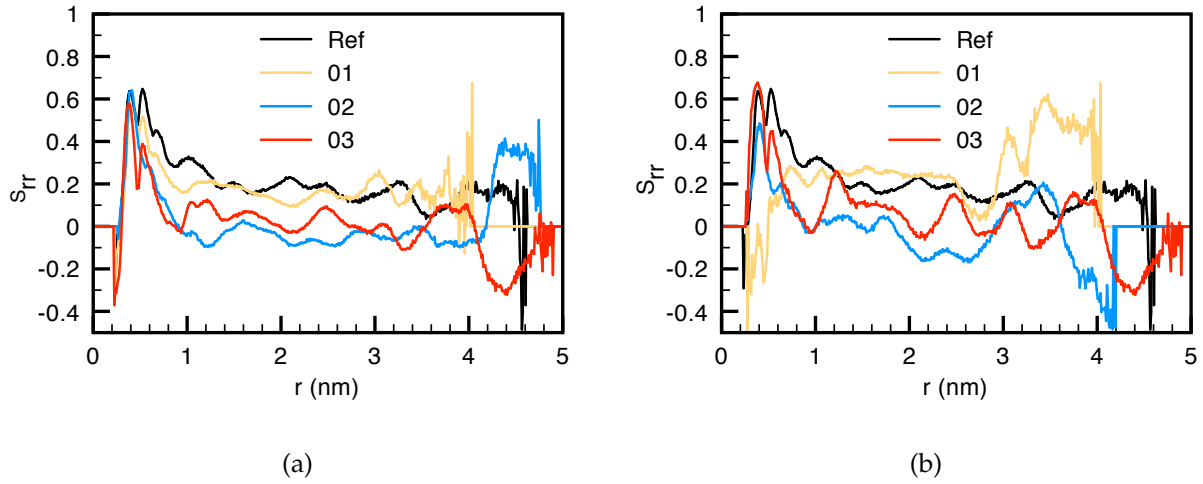


Figure 7: S_{rr} for all systems separately. (a) S_{rr} averaged over all CEs and BODIPY-CEs in each system, describing the average behavior around a ring structure. (b) S_{rr} calculated only around the BODIPY-CEs, describing the behavior around those. The reference (black curve) is the same in (a) and (b). r is the distance between the centers of the directors.

displacement analysis was performed so that for each BODIPY-CE a corresponding reference molecule was chosen with a location at a similar distance from the droplet center-of-mass as the BODIPY-CE in question.

Displacement distributions were constructed for BODIPY-CE using different time intervals (ranging from 1 ns to about 50 ns). The values for the diffusion coefficients converge to fixed values well before the estimated limit of 75 ns. In Figure 8 examples of jump length distributions together with the fitted diffusion models are shown. The diffusion coefficients computed by fitting the diffusion models to the distributions are given in Table 1 together with the diffusion model used for a given molecule type. To be able to compare diffusion coefficients obtained with the two- and three-dimensional diffusion models the, a scaled diffusion coefficient D_0 is used. From the definition of the diffusion equation (31) it can be seen that D_0 can be calculated from the coefficient for two and three dimensions by multiplying it by $2d$, where d is the number of dimensions.

Table 1: Diffusion coefficients in units of $10^{-7} \text{ cm}^2/\text{s}$

System	Molecule type	Dimensionality	D	D_0
Reference	CE	2D	0.59	2.36
01-03	CE	2D	0.57	2.28
	BODIPY-CE	2D	0.25	1.0
02	CE	2D	0.33	1.32
	BODIPY-CE	3D	0.16	0.96

The diffusion in the systems 01 and 03 follows the two-dimensional model. In these systems the analysed molecules are located at the interface, which explains the two-dimensional diffusion. The analysed molecules in the system 02 are located in the core

region where diffusion is expected to be three-dimensional. The better fitting of the two-dimensional model in the case of CEs in the system 02 might be explained by local ordering. It is possible that the CEs are locally in lamellar structure and that might explain two-dimensional diffusion locally. The number of analysed molecules per system is low and is prone to artifacts. The diffusion in the reference system follows the two-dimensional diffusion model because the core is small and most of the CEs are thus located close to the interface.

BODIPY-CE molecules in all systems seem to diffuse at a similar rate. The diffusion of the BODIPY-CE in the system 02 matches well with the diffusion of the chosen reference CE molecules. In the case of the systems 01 and 03 the rate of diffusion is half of that of the chosen reference molecules. The chosen reference molecules in the systems 01 and 03 are diffusing similarly to the CE molecules in the reference system. In an earlier study values at a similar range were obtained and were found to be in good agreement with the available experimental data (32). There are many factors affecting the rate of diffusion and some of them are discussed next. As seen earlier in this study, see Section 3.4, the packing of cholesterol ring structures around BODIPY-CE molecules is less ordered than around CE molecules, thus making it easier for BODIPY CE to diffuse in less ordered environment. On the other hand, the probe part of BODIPY-CE is bulkier than the short acyl chain of CE, hindering the diffusion. The BODIPY probe is slightly polar, whereas pure CE is apolar, and might interact more with water molecules and thus it might make them move slower. In the systems 01 and 03 the BODIPY-CE molecules are located closer to the lipid-water interface than in the system 02. The BODIPY-CE in the system 02 (closer to the core) prefer more extended conformations (as seen in Section 3.3) than the reference molecules, which might result in less entanglement effects that would slow down the diffusion.

4 Discussion

In this article, we have considered a new BODIPY containing cholesteryl oleate analog in a high-density lipoprotein environment. In particular, we have studied the behavior of BODIPY-cholesteryl oleate compared to normal cholesterol and also examined the disturbances that BODIPY-cholesteryl oleate molecules induce.

Fluorophores have an important role in experimental studies aimed to track the movements of different molecules between different compartments of a cell or to track intercellular trafficking. Both NBD and DHE fluorophores have been used for cholesterol tracking, and lately a BODIPY fluorophore has gained popularity due to its promising features (8). Recently a new cholesterol analog that includes the BODIPY-probe has shown superior features compared to other alternatives (9, 10). With the present study we take that structure as a basis and construct a new BODIPY containing cholesteryl oleate analog. We studied its behavior in a high-density lipoprotein environment and compared that with

the normal cholesteryl oleate. Our findings support the promising features seen in earlier studies: the behavior is close to normal cholesteryl oleate and the induced disturbances are small. The results indicate that BODIPY-cholesteryl oleate could be used as a viable probe in lipoprotein systems.

We found out that the distribution of BODIPY-cholesteryl oleate corresponds closely to the distribution of normal cholesteryl oleate. There are also only minor differences in the intramolecular conformations of BODIPY- and normal cholesteryl oleate; BODIPY containing cholesteryl oleates slightly favor more extended conformations. Both BODIPY- and normal cholesteryl oleate induce stacking around themselves with the difference that BODIPY-cholesteryl oleate's ability to induce stacking is somewhat diminished. And finally, also the dynamics of BODIPY-cholesteryl oleate seem to be in accordance with the normal cholesteryl oleate in the core region, but slower at the interface region.

Summarizing, the present results show that BODIPY-cholesteryl oleate mimics well the behavior of normal cholesteryl oleate in a lipoprotein environment and seems to be a suitable alternative for trafficking cholesteryl oleate in biological systems. More studies on the topic are though called for to better characterize the behavior on longer length and time scales.

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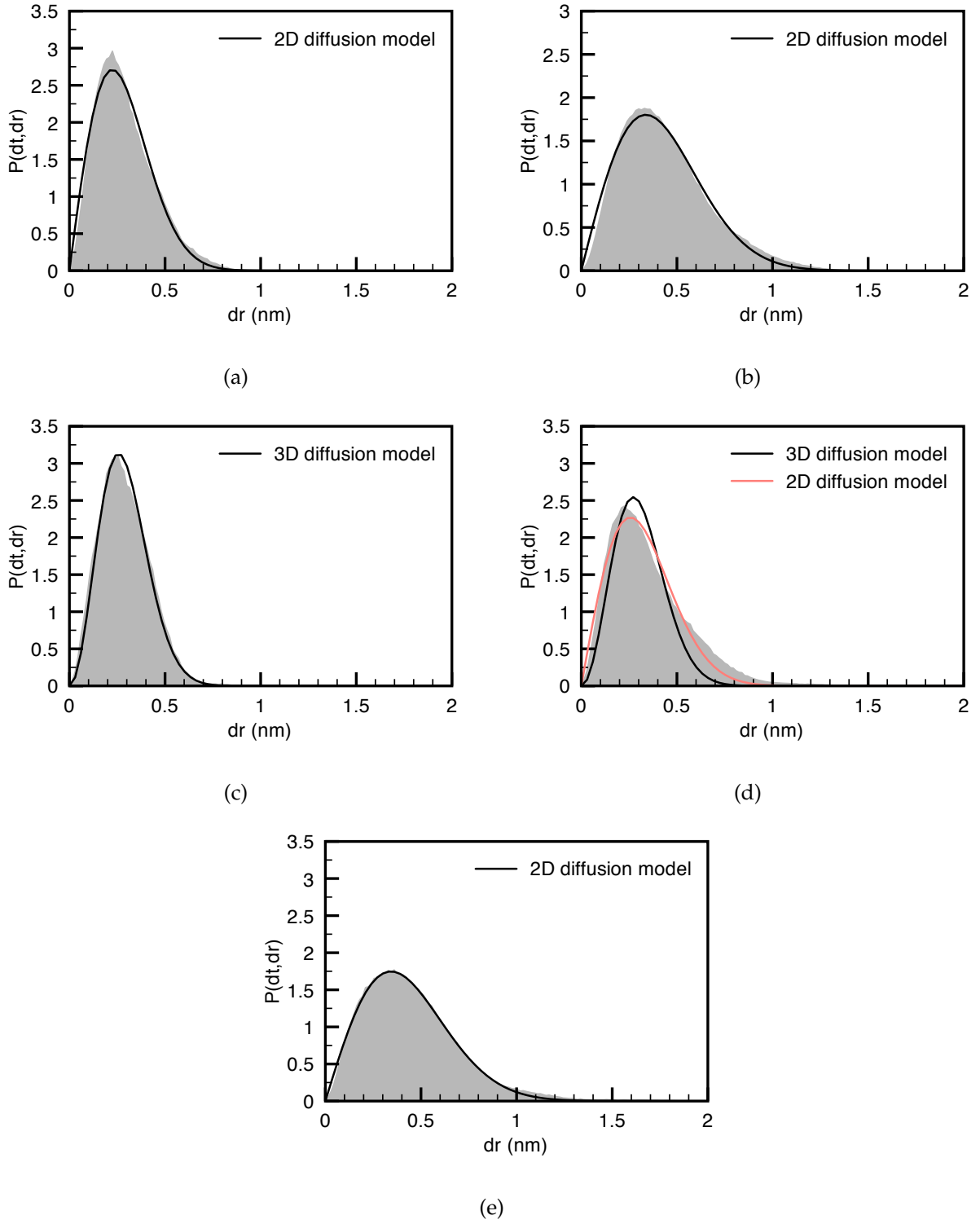


Figure 8: Examples of jump length distributions. (a) BODIPY-CE averaged over the systems 01 and 03. (b) Chosen reference CEs averaged over the systems 01 and 03. (c) BODIPY-CE in the system 02. (d) Chosen reference CEs in the system 02. (e) CE in the reference system. Gray color represents the jump length distributions with time interval of 10 ns. Black curve shows the diffusion model fitted to the jump length distribution. Red curve in panel (d) shows the alternative diffusion model fitted to the distribution, see text for details.

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